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<b>(54) Title: MODIFICATION OF STARCH SYNTHESIS IN PLANTS</b>  <b>(57) Abstract</b>  Plants, particularly cereal plants which have modifications to their starch synthesising pathway contain a DNA specifying the enzyme soluble starch synthase which has the sequence SEQ ID NO 1 or SEQ ID NO 2 or SEQ ID NO 3. The inserted gene may be inserted in a sense or anti-sense construct. The alteration introduced by the inserted genes may be a greater or reduced ability to produce starch or starch which has a different fine structure such as a different pattern of branching.		

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## MODIFICATION OF STARCH SYNTHESIS IN PLANTS

5 This invention relates to the alteration of the biosynthetic pathway which leads to production of starch in plants. By the term "alteration" we mean a change from normal of the amount or quality of the starch which the plant produces. More particularly, the invention relates to the isolation, purification and characterisation of the DNAs encoding several forms of the enzyme soluble starch synthase and the use of those DNAs through genetic modification of the plant genome to alter the starch production.

10 The invention also relates to novel plants having an improved ability to produce starch including an improved ability to produce structurally-altered starch.

Our previous studies have led to a new understanding of the metabolic pathway of starch synthesis in developing starch storing tissues (Keeling et al, 1988, Plant Physiology, 87:311-319; Keeling, 1989, ed. C.D. Boyer, J.C. Shannon and R.C. Harrison; pp.63-78, being a presentation at the 4th Annual Penn State Symposium in Plant Physiology).

15 Starch is an important end-product of carbon fixation during photosynthesis in leaves and is an important storage product in seeds and fruits. In economic terms, the starch produced by the edible portions of three grain crops, wheat, rice and maize, provide approximately two-thirds of the world's food calculated as calories.

20 Starch is synthesised in the plastid compartment, the chloroplast, in photosynthetic cells or the amyloplast in non- photosynthetic cells. The biochemical pathway of starch biosynthesis in leaves has been well-characterised (Figure 1). In contrast, little is known of the pathway of starch biosynthesis in storage organs.

25 Two principal methods for the control of gene expression are known. These are referred to in the art as "antisense downregulation" and "sense downregulation" or "cosuppression". Both of these methods lead to an inhibition of expression of the target gene. Overexpression is achieved by insertion of one or more than one extra copies of the selected gene. Other lesser used methods

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involve modification of the genetic control elements, the promoter and control sequences, to achieve greater or lesser expression of an inserted gene.

In antisense downregulation, a DNA which is complementary to all or part of the target gene is inserted into the genome in reverse orientation and without its translation initiation signal. The simplest theory is that such an antisense gene, which is transcribable but not translatable, produces mRNA which is complementary in sequence to mRNA product transcribed from the endogenous gene: that antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence: a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 40 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence.

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one plant species to be effective in another and obviates the need to construct antisense vectors for each individual species of interest. Although sequences isolated from one species may be effective in another, it is not infrequent to find exceptions where the degree of sequence similarity between one species and the other is insufficient for the effect to be obtained. In such cases, it may be necessary to isolate the species-specific homologue.

Antisense downregulation technology is well-established in the art. It is the subject of several textbooks and many hundreds of journal publications. The principal patent reference is European Patent No. 240,208 in the name of Calgene Inc. There is no reason to doubt the operability of antisense technology. It is well-established, used routinely in laboratories around the world and products in which it has been used are on the market.

Both overexpression and downregulation are achieved by "sense" technology. If a full length copy of the target gene is inserted into the genome then a range of phenotypes is obtained, some overexpressing the target gene, some underexpressing. A population of plants produced by this method may then be screened and individual phenotypes isolated. As with antisense, the inserted sequence is lacking in a translation initiation signal. Another similarity with antisense is that the inserted sequence need not be a full length copy. Indeed, it has been found that the distribution of over- and under- expressing phenotypes is skewed in favour of underexpression and this is advantageous when gene inhibition is the desired effect. For overexpression, it is preferable that the inserted copy gene retain its translation initiation codon. The principal patent reference on cosuppression is European Patent 465,572 in the name of DNA Plant Technology Inc. There is no reason to doubt the operability of this technology. It is well-established, used routinely in laboratories around the world and products in which it has been used are on the market.

Sense and antisense gene regulation is reviewed by Bird and Ray in *Biotechnology and Genetic Engineering Reviews* 9: 207-227 (1991). The use of these techniques to control selected genes in tomato has been described by Gray et.al., *Plant Molecular Biology*, 19: 69-87 (1992).

Gene control by any of the methods described requires insertion of the sense or antisense sequence, with appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of available methods.

For dicotyledonous plants the most widely used method is *Agrobacterium*-mediated transformation. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium *Agrobacterium tumefaciens*, or the related *Agrobacterium rhizogenes*, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by *Agrobacterium* in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

The effectiveness of *Agrobacterium* is restricted to the host range of the microorganism and is thus restricted more or less to dicotyledonous plant species. In general monocotyledonous species, which include the important cereal crops, are not amenable to transformation by the *Agrobacterium* method. Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

In summary, then, the requirements for both sense and antisense technology are known and the methods by which the required sequences may be introduced are

known. What remains, then is to identify genes whose regulation will be expected to have a desired effect, isolate them or isolate a fragment of sufficiently effective length, construct a chimeric gene in which the effective fragment is inserted between promoter and termination signals, and insert the construct into cells of the target plant species by transformation. Whole plants may then be regenerated from the transformed cells.

An object of the present invention is to provide DNAs encoding soluble starch synthases.

An further object of the invention is to provide novel plants having an increased capacity to produce starch and a capacity to produce starch with an altered fine structure.

According to the present invention there is provided cDNAs having the sequences of the inserts in plasmids pSSS6, pSSS10.1 and pSSS6.31 and sequences having sufficient similarity such that when inserted into the genome of an organism which produces starch, the synthesis of starch is altered.

The plasmid pSSS6 was deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 13th June 1994, under the Accession Number 40651.

The plasmids pSSS6.31 and pSSS10.1 were deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 22nd August 1994, under the Accession Numbers NCIMB 40679 and 40680 respectively.

The invention also provides the cDNAs, encoding soluble starch synthases which have the sequences SEQ-ID-NO-1, SEQ-ID-NO-2 AND SEQ-ID-NO-3.

The invention also provides transformed plants containing one or more copies of one or more of the said cDNAs in sense or antisense orientation. The description which follows will describe a method for the isolation of the genes encoding soluble starch synthases from maize.

These DNAs can be used for the isolation of the corresponding genomic sequences. Either the cDNAs or the genes can then be used in studies leading to the

increase in starch yield. One possible application could be the use of these sequences to increase gene dosage of SSS in transformed crop plants to determine the contribution of SSS to the net regulation of starch biosynthesis, and to modify the levels of starch synthesised by the plant. The introduction of additional copies of SSS genes should produce greater levels of the enzyme in the amyloplasts.

Increased gene expression may also be elicited by introducing multiple copies of enhancer sequences into the 5'-untranscribed region of SSS gene. If the enzyme is rate-limiting to starch biosynthesis, then the rate of starch biosynthesis would be expected to increase in the transformed plants. By virtue of this invention it will also be possible to alter the kinetic properties of the endopserm enzyme through protein engineering. Obviously a number of other parameters could also be improved. The present invention will now be described, by way of illustration, by the following Example and with reference to the accompanying drawings of which:

Figure 1 shows the reactions involved in the biosynthetic pathways of starch and glucose in leaves. The abbreviations used are: G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; Pi, orthophosphate; PPi, inorganic pyrophosphate. The reactions are catalysed by the following enzymes:

- 1) phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase
- 2) triose-phosphate isomerase
- 3) aldolase
- 4) fructose-1,6-bisphosphatase
- 5) hexose phosphate isomerase
- 6) phosphoglucomutase
- 7) ADP-glucose pyrophosphorylase
- 8) starch synthase
- 9) UDP-glucose pyrophosphorylase
- 10) sucrose phosphate synthase
- 11) sucrose phosphatase
- 12) orthophosphate/triose phosphate translocator
- 13) inorganic pyrophosphatase



Figure 2 shows the proposed metabolic pathway of starch biosynthesis in wheat endosperm (Keeling et. al. 1988). The abbreviations used are the same as in Figure 1. The reactions are catalysed by the following enzymes:

- 5                   1)       sucrose synthase
- 2)       UDP-glucose pyrophosphorylase
- 3)       hexokinase
- 4)       phosphoglucomutase
- 10               5)       hexose-phosphate isomerase
- 6)       ATP-dependent phosphofructokinase
- 7)       PPi-dependent phosphofructokinase
- 8)       aldolase
- 9)       triose-phosphate isomerase
- 15               10)      hexose-phosphate translocator
- 11)      ADP-glucose pyrophosphorylase
- 12)      starch synthase
- 13)      sucrose phosphate synthase
- 14)      sucrose phosphatase

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#### USE OF SOLUBLE STARCH SYNTHASE OR BRANCHING ENZYME

Using standard cloning techniques, the SSS genes may be isolated. The source of the genes was a US yellow-dent corn line of *Zea mays*, from which the enzyme protein was purified.

Endosperms from the maize line were homogenised in a buffer which maintains the SSS in active  
25   form.

Purification of the SSS from maize has been achieved by a combination of ammonium sulphate precipitation, DEAE-cellulose chromatography, gel-filtration, phenyl Superose and FPLC using a Mono-Q column. This results in several hundred-fold purification with yields up to 5%. The SSS polypeptide was a single subunit of molecular weight 76kDa. Other SSS polypeptides  
30   were present in a US dent inbred line at around 60kDa, 70kDa and 105kDa molecular weight.

Ammonium sulphate precipitation of SSS I is best achieved using 10-35% ammonium sulphate which produces a translucent SSS-enriched pellet which is next dialysed and further fractionated using DEAE-cellulose ion-exchange chromatography (2.5 x 5cm column). SSS was eluted with a 150 ml gradient of KCl (0-0.6M) and fractions collected. These steps increase specific activities by up to 12-fold. The DEAE peak fractions were concentrated by precipitation with ammonium sulphate (40%) and the resulting pellet dissolved in buffer and fractionated on a Sephacryl S-200 column (2.5 x 100 cm) equilibrated with buffer and fractions collected. These steps increase specific activities by up to 8-fold. A Phenyl-Superose column was equilibrated with buffer containing ammonium sulphate. SSSI did not bind and was present in the pass-through fraction. These steps increase specific activities by up to 2-fold. Finally, a Mono-Q column was equilibrated with buffer and charged with the Phenyl-Superose pass-through fraction. The enzymes were eluted from the column using a 12 ml linear gradient of 0-0.5 M KCl and fractions collected. These steps increase specific activities by up to 5-fold.

In the final purification step the SSS preparations were loaded on to SDS PAGE gels. The bands corresponding to the SSS polypeptides were cut out and eluted. The polypeptide was sequenced using standard amino acid sequencing techniques.

In order to produce a pure antigen for antibody production, we decided to use starch granules as our starting-point for isolation of SSS proteins. Kernels were homogenised in buffer by grinding in a Waring blender. The homogenate filtered through miracloth and centrifuged. After discarding the supernatant and the discoloured material that overlays the white starch pellet, the pellet was washed twice with buffer and centrifuged. Starch was washed a final time with chilled acetone and following centrifugation, dried under a stream of air before storing at -20C. Granule protein was extracted by boiling 1.4 g starch for 10 minutes in 50ml SDS-PAGE sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 62.5 mM Tris/HCl, pH 6.8) which lacked bromophenol blue. After cooling and centrifugation at 25,000 g at 4C for 15 minutes, the supernatant was mixed with an equal volume of 30% TCA and allowed to stand at 4C for 1 hour. The solution was centrifuged again and pellet washed twice with 10 ml acetone before resuspension in 1.4 ml SDS-PAGE sample buffer. Following separation of granule-derived proteins by SDS-PAGE, the SSS proteins (eg 60kDa, 76kDa etc) bands were electroeluted and used as antigen (three 50ug doses at 4-week intervals, in New Zealand white rabbits) to generate polyclonal antibodies in a rabbit. The antibodies were then tested for specificity to the SSS

polypeptides. Antibodies were monospecific and have enabled a thorough analysis of enzyme activities and expression studies.

N-terminal amino acid sequences were also obtained from the polypeptides. These proteins were shown to be identical with soluble proteins on the basis of (i) N-terminal sequences to the SSSs as purified by conventional means and sequenced were identical to the granule derived proteins, and (ii) protease digests gave peptide maps which were also identical.

Amino acid sequencing of the maize SSS polypeptide has yielded the following partial sequences:

N-terminal... CVAELSREGPAPR

Internal sequences:

10        KNYANAFYTETHI  
          ELGGYIYGQNDMFVVNNDHASLVPVLLAAKYIR  
          EVTTAEGGSGLNELL  
          GKIDNTVVVASEQDSY

15        The antibodies may be used to screen a maize endosperm cDNA library for clones derived from the mRNAs for SSS in an in vitro transcription/ translation system. Synthetic oligos may be constructed and used to screen maize endosperm cDNA library. The SSS sequence may be compared to the amino acid sequence of pea SSS I and SSS II published by Dry et al (1991, Plant Journal 2:193-202) or rice SSS published by Baba et al (1993, Plant Physiology 103, 565-573).  
20        Interestingly, the clone obtained from rice SSS is not correctly identified. The N-terminal sequence AELSREG is stated to be part of the transit peptide sequence of the rice clone. This error must have occurred because of protein isolation problems from rice kernels: presumably a portion of the protein was cleaved prior to isolation. Using our N-terminal sequence, the corrected molecular weight of the rice clone is around 69kDa and not 55 or 57kDa as suggested by Baba et al.

### cDNA LIBRARY SCREENING AND ISOLATION OF SSS cDNA CLONES

RNA was extracted from from 21 DAP endosperm (obtained from the inbred line B73) after removal of pericarp and embryo. The library consisted of ~900,000 recombinant clones. A probe for granule bound starch synthase was generated using PCR and used to screen an aliquot of the library, ~500,000 recombinants. This screening yielded approximately 200 positive signals. Isolation and sequencing of a number showed them to be full length GBSS cDNA clones.

An oligonucleotide was synthesised to N-terminal sequence obtained from the purified SSS protein and used to screen the same aliquot of library as that used for the GBSS screening. No positive signals were obtained. A long oligonucleotide probe was then synthesised to the ADP-ADPG binding region and following sequence, based on a comparison of the sequences published for pea SSS, rice SSS and maize GBSS.

The sequence of the oligonucleotide was GGT/C GGA/G CTA/T GGAGATGTTTGTGGA/T TCACTCCCAATTGCTCTT/G GCTCTTCGTGGA/T CATCGTGTG/T ATGGTTGT. Fifteen strong signals were obtained, all were picked, of these ten plaque purified after two rounds of purification. Restriction analysis of all ten showed them to fall into two classes. Sequence analysis showed both classes to be starch synthases.

Screening of a maize seedling library (Clontech) gave positive signals using 5' probes from one class of clones only.

A cDNA library from the inbred line W64A was screened and full length clones were isolated as judged by comparison with N-terminal sequence.

### CHARACTERISATION OF cDNA CLONES

The isolated cDNAs were sequenced and are given herewith as SEQ-ID-NO-1, NO-2 and NO-3.

For comparison, the deduced amino acid sequences are shown here with the sequences obtained directly from the protein:-

CVAELSREGPAPR

peptide derived

CVAELSREGPAPR

deduced cDNA

KXYANAFYTETHI

peptide derived

KNYANAFYSEKHI

deduced cDNA

10.52

EVTTAEGGSGLNELL

peptide derived

EVTTAEGGQGLNELL

deduced cDNA 10.52

ELGGYIYGANXMFVVNXXHASLVPVLLAAKY

peptide derived

5 ELGGYIYGQNCMLVVNDWHASLEPVLLAAKY

deduced cDNA 10.52

GKIDNTVVVASEQDSY

peptide derived

GSIDNTVVVASEQDSE

deduced cDNA 10.52

10

Isolated from soluble 76kDa protein.....

GLVVTRDRDRIQ-VASNR

peptide derived

GAVVTADRIVTVSKGYS

deduced cDNA

10.52

- 15 Clone SSS6.31 contained none of these internal sequences. The motif for the binding-site of ADPG and ADP, thought to be part of the active site of starch synthases is found in all clones near to the 5' end and is followed by the highly conserved sequence on which the oligonucleotide probe was based. The highly conserved domain SRFEPGLNQLYAMXYGTXXXXXXXXXGGLRDTV is present in SSS10.52 but is slightly
- 20 modified in SSS6.31 in that the EPC motif is replaced with an AG motif.

#### Expression of maize starch synthases in Escherichia coli BL21(DE3).

These SSS clones have been transfected into E.coli. The SSS activity was measured and are reported in the Table below.

Plasmids	Maize starch synthase genes	N-terminus	Protein	Specific

			(mg/mL)	Activities* (units/mg Protein)
pET21a	Native plasmid	<no insert>	1.8	.009
pEXS-3a	MSSSII (MSSS631)	GENVMNVIV V	2.8	0.069
pEXS-8	MSSSI (MSSS6- 4)	CVAELSREGP	1.9	0.097
pEXS-9		GSVGAALRSY	1.8	0.515
pEXS-wx	MSSSIII (MSSS5.6)	ASAGMNVVF V	2.0	0.033
	MGBSS (waxy)			

- One unit activity is defined as one mmol glucose incorporated into  $\alpha$ -1,4 glucan per minute at 25°C using 5 mg/mL glycogen as primer.

#### GENE CONSTRUCTS FOR TRANSFORMATION

5 The gene constructs require the presence of an amyloplast transit peptide to ensure its correct localisation in the amyloplast. It is believed that chloroplast transit peptides have similar sequences but other potential sources are available such as that attached to ADPG pyrophosphorylase (Plant Mol. Biol. Reporter (1991) 9, 104-126). Other potential transit peptides are those of small subunit RUBISCO, acetolactate synthase, glyceraldehyde-3P-dehydrogenase and nitrite reductase. For example,

10 Consensus sequence of the transit peptide of small subunit RUBISCO from many genotypes has the sequence:

MASSMLSSAAV $\frac{1}{2}$ ATRITNPAQAS      MVAPFTGLKSAAFPVSRK      QNLDITSIA  
SNGGRVQC

15 and the corn small subunit RUBISCO has the sequence:

MAPTVMMASAT-ATRITNPAQAS      AVAPFQGLKSTASLPVARR      SSRSLGNVA  
SNGGRIRC

The transit peptide of leaf starch synthase from corn has the sequence:

20 MA ALATSQVLVAT RAGLGVPDAS TFRRGAAQGL RGARASAAAD TLSMRTASARA  
APRHQQQARR GGRFPSLVVC

The transit peptide of leaf glyceraldehyde-3P- dehydrogenase from corn has the sequence:

MAQILAPS TQWQMRITKT SPCATPITSK MWSSLVMKQT KKVAHSAKFR  
VMAVNSENGT

The putative transit peptide from ADPG pyrophosphorylase from wheat has the sequence:  
RASPPSESRA PLRAPQRSAT RQHQRQGP RMC

- 5 It is possible however to express the genes constitutively using one of the well-known constitutive promoters such as CaMV35S but there may be biochemical penalties in the plant resulting from increased starch deposition throughout the entire plant. Deposition in the endosperm is much preferred.
- 10 Possible promoters for use in the invention include the promoters of the starch synthase gene, bound starch synthase gene, endosperm hsp70 gene, ADPG pyrophosphorylase gene, and the sucrose synthase gene.

**FOR TESTING GENE EXPRESSION IN ENDOSPERM TISSUE:**

Plasmid name	Promoter	Intron	Targetting	Gene
pHKH1	CaMV35S	adh1	WxTrPep	GUS
pSh1PIGN	CaMV35S	adh1	WxTrPep	GUS
pSh2PIGN	CaMV35S	adh1	WxTrPep	GUS

**FOR TESTING IN SUSPENSION CELL CULTURES:**

Plasmid name	Promoter	Intron	Targetting	Gene
p***1	CaMV35S	Sh1	WxTrPep	GUS
p***2	CaMV35S	adh1	WxTrPep	GUS

**FULL VECTORS FOR PLANT TRANSFORMATION**

Plasmid name	Promoter	Intron	Targetting	Gene
p***21	Waxy	Sh1	WxTrPep	SSS and/or BE
p***22	Waxy	Adh1	WxTrPep	SSS and/or BE
p***23	Sh1	Sh1	WxTrPep	SSS and/or BE
p***24	Sh1	Adh1	WxTrPep	SSS and/or BE
p***25	Sh2	Sh1	WxTrPep	SSS and/or BE
p***26	Sh2	Adh1	WxTrPep	SSS and/or BE
p***27	hsp70	Sh1	WxTrPep	SSS and/or BE
p***28	hsp70	Adh1	WxTrPep	SSS and/or BE

## TRANSFORMATION

### (i) Insertion of extra copies of the gene

Maize genomic DNAs isolated as above may subsequently be transformed into either  
5 protoplasts or other tissues of a maize inbred line or population. The existing gene promoters ensure that the extra genes are expressed only in the developing endosperm at the correct developmental time. The protein sequences likewise ensure that the enzymes are inserted into the amyloplast.

Transgenic maize plants are regenerated and the endosperms of these plants are tested for  
10 increased SSS enzyme activity. The kernels are also tested for enhanced rate of starch synthesis at different temperatures. The plants are then included in a breeding programme to produce new maize hybrids with higher rates of starch synthesis at temperatures above the normal optimum.

### (ii) Insertion of genes specifying SSS

15

This is also achieved by standard cloning techniques. The source of the temperature-stable forms of the SSS genes is any organism that can make starch or glycogen. Potential donor organisms are screened and identified as described above. Thereafter there are two approaches:

- (a) via enzyme purification and antibody/sequence generation using the protocol described  
20 above.
- (b) using SSS cDNAs as heterologous probes to identify the genomic DNAs for SSS in libraries from the organism concerned. The gene transformation, plant regeneration and testing protocols are as described above. In this instance it is necessary to make gene constructs for transformation which contain the regulatory sequences from maize endosperm SSS or  
25 another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct developmental time (eg, ADPG pyrophosphorylase).

Gene constructs used to transform plants requires the regulatory sequences from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct development time (eg, ADPG pyrophosphorylase).



Furthermore the gene constructs also requires a suitable amyloplast transit-peptide sequence such as from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to ensure expression of the amyloplast at the correct developmental time (eg, ADPG pyrophosphorylase).

- 5            Genetic protein engineering techniques may also be used to alter the amino acid sequence of the SSS enzymes to impart higher temperature optima for activity. The genes for SSS may be cloned into a bacteria which relies on these enzymes for survival. Selection for bacteria surviving at evaluated temperatures enables the isolation of mutated thermostable enzyme forms. Transformation of maize with the altered genes is carried out as described above.

10

(iii)    Changing the ratios of activities of the isoforms of enzymes SSS

          This is also achieved by standard cloning techniques. The source of the SSS genes is maize using the protocol described above. Plants are then transformed by insertion of extra gene  
15    copies of the isoforms of SSS enzymes and/or by insertion of anti- sense gene constructs. The gene promoters and other regulatory sequences may also be altered to achieve increased amounts of the enzyme in the recipient plant.

(iv)    Insertion of a gene or genes specifying SSS with activities which effect a change in the fine structure of the starch.

20

          This is also achieved by standard cloning techniques. The source of the special forms of the SSS is any organism that can make starch. Potential donor organisms are screened and identified as described above. Thereafter there are two approaches:

- (a)    via enzyme purification and antibody/sequence generation using the protocol described  
25    above.
- (b)    using SSS cDNAs as heterologous probes to identify the genomic DNAs for SSS in libraries from the organism concerned. The gene transformation, plant regeneration and testing protocols are as described above. In this instance it is necessary to make gene

- 16 -

constructs for transformation which contain the regulatory sequences from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct developmental time (eg, ADPG pyrophosphorylase).

**Full length clone sequences**

SEQ-ID-NO1; DNA; 2992 BP.

CC NOTE: ORIGINAL SEQUENCE NAME WAS SSS1052 and SSS64

SQ SEQUENCE 2992 BP; 758 A; 655 C; 801 G; 776 T; 2 OTHER;

5       GAATTCGCGG CCGCCTTATT TCTGGTTGGC CACATACATC ATCCAAAAAA  
      CTTTATTATT

      GAATTACAAC TAATAAGCAA TCTAAAAGAG GGCACCACCA ATGATGTGTT  
      GTTGGTAGGA

10       GGCCGCTGGG TCTGTCAAAG CAAGTTGGAC AAAGGGCAAC AATTGTTGTA  
      GTTGTAAGAG

      GGTTGCGGGG TTAGCCGCAA ACTGCTGGTA GAAAGGCAGC AACTGTTGCT  
      GTGTCAAGAA

      GGAAGCACGG TTTGCTGCAG CTGTTGTGCC CTGATGGTTT GTACCAATGA  
      CTGCACCAAA

15       GATAGGGCTG GCGATTGTTG AAACAACAAG GCGGATAAAG GTATGTTGCT  
      TGCTGCGATT

      GCTTGTTGAA GCCTATATGG TTGAAGAGCT GGGTTTTTAC ATATTGAAGC  
      TATAATTGAT

20       GGAAGGTATG GGGGAAGAAG GGAAGCTATA GGAGCTTGTG AGCATTGAGG  
      GAAAATTGTC

      GCGTTAGCAA CACATGTAGA AAGAGCAAGG AGCATAAGGA GGGAAAATAT  
      CTTGGTCGCC

      ATTGTTGCGC GCGATCCACG GCCCCCCCCC CCCGCGCTCC TGTCTGCTCT  
      CCCTCTCCGC

25       AATGGCGACG CCCTCGGCCG TGGGCGCCGC GTGCCTCCTC CTCGCGCGGG  
      NCG CCTGGCC

      GGCCGCCGTC GGCGACCGGG CGCGCCCGCG GAGGCTCCAG CGCGTGCTGC  
      GCCGCCGGTG

CGTCGCGGAG CTGAGCAGGG AGGGGCCCCG GCCGCGCCCCG CTGCCACCCG  
CGCTGCTGGC

GCCCCGCTC GTGCCCCGGCT TCCTCGCGCC GCCGGCCGAG CCCACGGGTG  
AGCCGGCATC

5 GACGCCGCCG CCCGTGCCCCG ACGCCGGCCT GGGGGACCTC GGTCTCGAAC  
CTGAAGGGAT

TGCTGAAGGT TCCATCGATA ACACAGTAGT TGTGGCAAGT GAGCAAGATT  
CTGAGATTGT

10 GGTTGGAAAG GAGCAAGCTC GAGCTAAAGT AACACAAAGC ATTGTCTTTG  
TAACCGGCGA

AGCTTCTCCT TAATCGAAAG TCTGGGGGTC TAGGAGATGT TTGTGGTTCA  
TTGCCAGTTG

CTCTTGCTGC TCGCGGTCAC CGTGTGATGG TTGTAATGCC CAGACATTTA  
AATGGTACCT

15 CCGATAAGAA TTATGCAAAT GCATTTTACT CAGAAAAACA CATTCCGATT  
CCATTCTTTG

GCGGTGAACA TGAAGTTACC TTCTTCCATG AGTATAGAGA TTCAGTTGAC  
TGGGTGTTTG

20 TTGATCATCC CTCATATCAC AGACCTGGAA ATTTATATGG AGATAAGTTT  
GGTGCTTTTG

GTGATAATCA GTTCAGATAC AACTCCTTT GCTATGCTGC ATGTGAGGCT  
CCTTTGGTCC

TTGAATTGGG AGGATATATT TATGGACAGA ATTGCATGTT GGTTGTCAAT  
GATTGGCATG

25 CCAGTCTAGA GCCAGTCCTT CTTGCTGCAA AATATAGACC ATATGGTGTT  
TATAAAGACT

CCCGCAGCAT TCTTGTAATA CATAATTTAG CACATCAGGG TGTAGAGCCT  
GCAAGCACAT

30 ATCCTGACCT TGGGTTGCCA CCTGAATGGT ATGGAGCTCT GGAGTGGGTA  
TTCCCTGAAT

GGGCGAGGAG GCATGCCCTT GACAAGGGTG AGGCAGTTAA TTTTTTGAAA  
GGTGCAAGTTG

TGACAGCAGA TCGAATCGTG ACTGTCAGTA AGGGTTATTC ATGGGAGGTC  
ACAACTGCTG

5 AAGGTGGACA GGGCCTCAAT GAGCTCTTAA GCTCCAGAAA GAGTGTATTA  
AACGGAATTG

TAAATGGAAT TGACATTAAT GATTGGAACC CTGCCACAGA CAAATGTATC  
CCCTGTCATT

10 ATTCTGTTGA TGACCTCTCT TGAAAGGCTA AATGTAAAGG TGCATTGCAG  
AAGGAGCTGG

GTTTACCTAT AAGGCCTGAT GTTCCTCTGA TTGGCTTTAT TGGAAGATTG  
GATTATCAGA

AAGGCATTGA TCTCATTCAA CTTATCATAC CAGATCTCAT GCGGAAGAAT  
GTTCAA TTG

15 TCATGCTTGG ATCTGGTGAC CCAGAGCTTG AAGATTGGAT GAGATCTACA  
GAGTCGATCT

TCAAGGATAA ATTTCTGTGA TGGGTTGGAT TTAGTGTTCC AGTTTCCCAC  
CGAATAACTG

20 CGGCTGGCGA TATATTGTGA ATGCCATCCA GATTCGAACC TTGTGGTCTC  
AATCAGCTAT

ATGCTATGCA GTATGGCACA GTTCCTGTTG TCCATGCAAC TGGGGGCCTT  
AGAGATACCG

TGGAGAACTT CAACCCTTTC GGTGAGAATG GAGAGCAGGG TACAGGGTGG  
GCATTCGCAC

25 CCCTAACCAC AGAAAACATG TTTGTGGACA TTGCGAACTG CAATATCTAC  
ATACAGGGAA

CACAAGTAAT AATGGGAAGG GCTAATGAAG CCAGGCATGT CAAAAGAGTT  
CACGTGGGAC

30 CATGCCGCTG AACAATACGA ACAAATCTTC CAGTGGGCCT TCATCGGATC  
GACCCGATGT

TCAATGGAAA AAAGGGACCA AAGTTGGTTG GTTCCTTGAA GATTATCAGT  
TCATCATCCT

ATAGTAAGCT GAATGATGAA AGAAAACCCC TGTACATTAC ATGGAAGGCA  
GACCGGCTAT

5 TGGCTCCATT GCTCCAATGT CTGCTTTGGC TGCCTTGCCT CGATGGACCG  
GATGCAGTGA

GGAATCCAGN CGAACGACAG TTTTGAAGGA TAGGAAGGGG AGCTGGAAGC  
AGTCACGCAG

10 GCAGGCAAGC CTTGCGCGTT AATTCATATG GAACAAGCTG GAGTCAGTTT  
CTGCTGTGCC

ACTCACTGTT TACCTTAAGA TTATTACCTG TGTTGTTCTC CTTTGCTCGT  
TAGGGCTGAT

AACATAATGA CTCATTAAGA ATATAATTCA CTCTGCCTCG TTTTAAATCT  
TAAGTGAAGT

15 CGAGATCTAC TTCGTCATTC CTTCCCCGTT TAAAAAAAAA AAAAAAAAAA AA

SEQ-ID-NO2; DNA; 2085 BP.

CC NOTE: ORIGINAL SEQUENCE NAME WAS SSS CLONE 6.31

20 SQ SEQUENCE 2085 BP; 456 A; 521 C; 629 G; 479 T; 0 OTHER;

AACGCCGCAT TGGCACGTTG AGATCAAGTC CATCGTCGCC GCGCCGCCGA  
CGAGCATAGT

GAAGTTCCCA GGGCGCGGGC TACAGGATGA TCCTTCCCTC TGGGACATAG  
CGCCGGAGAC

25 TGTCTCCCA GCCCGAAGC CACTGCATGA ATCGCCTGCG GTTGACGGAG  
ATTCAAATGG

AATTGCACCT CCTACAGTTG AGCCATTAGT ACAGGAGGCC ACTTGGGATT  
TCAAGAAATA

CATCGGTTTT GACGAGCCTG ACGAAGCGAA GGATGATTCC AGGGTTGGTG  
CAGATGATGC

TGGTTCTTTT GAACATTATG GGACAATGAT TCTGGGCCTT TGTGGGGAGA  
ATGTTATGAA

5 CGTGATCGTG GTGGCTGCTG AATGTTCTCC ATGGTGCAAA ACAGGTGGTC  
TTGGAGATGT

TGTGGGAGCT TTACCCAAGG CTTTAGCGAG AAGAGGACAT CGTGTTATGG  
TTGTGGTACC

10 AAGGTATGGG GACTATGTGG AAGCCTTTGA TATGGGAATC CGGAAATACT  
ACAAAGCTGC

AGGACAGGAC CTAGAAGTGA ACTATTTCCA TGCATTTATT GATGGAGTCG  
ACTTTGTGTT

CATTGATGCC TCTTTCCGGC ACCGTCAAGA TGACATATAT GGGGGAAGTA  
GGCAGGAAAT

15 CATGAAGCGC ATGATTTTGT TTTGCAAGGT TGCTGTTGAG GTTCCTTGGC  
ACGTTCCATG

CGGTGGTGTG TGCTACGGAG ATGGAAATTT GGTGTTTATT GCCATGAATT  
GGCACACTGC

20 ACTCCTGCCT GTTTATCTGA AGGCATATTA CAGAGACCAT GGGTTAATGC  
AGTACACTCG

CTCCGTCCTC GTCATACATA ACATCGGCCA CCAGGGCCGT GGTCCTGTAC  
ATGAATTCCC

GTACATGGAC TTGCTGAACA CTAACCTTCA ACATTTGAG CTGTACGATC  
CCGTCCGTGG

25 CGAGCACGCC AACATCTTTG CCGCGTGTGT TCTGAAGATG GCAGACCGGG  
TGGTGACTGT

CAGCCGCGGC TACCTGTGGG AGCTGAAGAC AGTGGAAGGC GGCTGGGGCC  
TCCACGACAT

30 CATCCGTTCT AACGACTGGA AGATCAATGG CATTCTGAA CGCATCGACC  
ACCAGGAGTG

GAACCCCAAG GTGGACGTGC ACCTGCGGTC GGACGGCTAC ACCAACTACT  
CCCTCGAGAC

ACTCGACGCT GGAAAGCGGC AGTGCAAGGC GGCCCTGCAG CGGGACGTGG  
GCCTGGAAGT

5 GCGCGACGAC GTGCCGCTGC TCGGCTTCAT CGGGCGTCTG GATGGACAGA  
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CATCATCGGG GACGCGATGC CGTGGATCGC GGGGCAGGAC GTGCAGCTGG  
TGATGCTGGG

10 CACCGGCCCA CCTGACCTGG AACGAATGCT GCAGCACTTG GAGCGGGAGC  
ATCCCAACAA

GGTGCGCGGG TGGGTCGGGT TCTCGGTCCT AATGGTGCAT CGCATCACGC  
CGGGCGCCAG

CGTGCTGGTG ATGCCCTCCC GCTTCGCCGG CGGGCTGAAC CAGCTCTACG  
CGATGGCATA

15 CGGCACCGTC CCTGTGGTGC ACGCCGTGGG CGGGCTCAGG GACACCGTGG  
CGCCGTTCGA

CCCGTTCGGC GACGCCGGGC TCGGGTGGAC TTTTGACCGC GCCGAGGCCA  
ACAAGCTGAT

20 CGAGGTGCTC AGCCACTGCC TCGACACGTA CCGAAACTAC GAGGAGAGCT  
GGAAGAGTCT

CCAGGCGCGC GGCATGTCGC AGAACCTCAG CTGGGACCAC GCGGCTGAGC  
TCTACGAGGA

CGTCCTTGTC AAGTACCAGT GGTGAACCCT CCGCCCTCCG CATCAATATC  
TTCGGTTTGA

25 TCCCATTGTA CATCGCCCTT TGACGGTCTC GGTGAAGAAC TTCATATGCA  
GTGCCGTGCT

GGGGCGGTAG CAGTACTATG GGATTGCATT GAGCTGTGTC ACTATGTGCT  
TTCGACAGGA

30 CAGTAGTGAA GGTTCATATGC AAGTTTATTT TTTTTTTCAT TACTGATATT  
TGGAATGTCA



ACACAATAAA TAACTACTAT GTGTTTCGTA AGTAAAAAAA AAAAA

SEQ-ID-NO3: 2478 bp DNA 04-DEC-1995

5 CC NOTE: ORIGINAL SEQUENCE NAME WAS SSS56

SUMMARY #Molecular-weight 89141 #Length 826 #Checksum 2983

BASE COUNT 347 A 276 C 533 G 290 T

ORIGIN

1 GCNGCNGCNT GGTRGCMYT NGTNCARGCN GARGCNGCNG TNGCNTRRGG  
10 NATHCCNATG

61 CCNCGGNGCNA THWSNWSNWS NWSNWSNGCN TTYTNYTNC CNGTNGCNWS  
NWSNWSNCCN

121 MGNMGNMGNM GNGGNWSNGT NGGNGCNGCN YTNMGNWSNT  
AYGGNTAYWS NGGNGCNGAR

15 181 YTNMGNYTNC AYTGGGCNMG NMGNGGNCCN CCNCARGAYG GNGCNGCNWS  
NGTNMGNGCN

241 GCNGCNGCNC CNGCNGGNGG NGARWSNGAR GARGCNGCNA ARWSNWSNWS  
NWSNWSNCAR

301 GCNCGGNGCNG TNCARGGNWS NACNGCNAAR GCNGTNGAYW SNGCNWSNCC  
20 NCCNAAAYCCN

361 YTNACNWSNG CNCCNAARCA RWSNCARWSN GCNGCNATGC ARAAYGGNAC  
NWSNGGNGGN

421 WSNWSNGCNW SNACNGCNGC NCCNGTNWSN GGNCNAARG CNGAYCAYCC  
NWSNGCNCCN

25 481 GTNACNAARM GNGARATHGA YGCNWSNGCN GTNAARCCNG ARCCNGCNGG  
NGAYGAYGCN

541 MGNCCNGTNG ARWSNATHGG NATHGCNGAR CCNGTNGAYG CNAARGCNGA  
YGCNGCNCCN

601 GCNACNGAYG CNGCNGCNWS NGCNCNTAY GAYMGNGARG AYAAYGARCC  
NGGNCCNYTN

661 GCNCGNCCNA AYG TNATGAA YGTNGTNGTN GTNGCNWSNG ARTGYGCNCC  
NTTYTGAAAR

5 721 ACNCGNGGNY TNGGNGAYGT NGTNGGNGCN YTNCCNAARG CNYTNGCNMG  
NMGNGGNCAAY

781 MGNGTNATGG TNGTNATHCC NMGN TAYGGN GARTAYGCNG ARG CNMGNGA  
YYTNGGNGTN

10 841 MGNMGNMGNT AYAARGTNGC NGGNCARGAY WSN GARGTNA CNTAYTTYCA  
YWSNTAYATH

901 GAYGGNGTNG AYT TYGTNTT YGTNGARGCN CCNCCNTTYM GNCA YMGNCA  
YAAYAAYATH

961 TAYGGNGGNG ARMGN YTN GA YATHYTNAAR MGNATGATHY TTTYTGAA  
RGCNGCNGTN

15 1021 GARGTNCCNT GGTAYGCNCC NTGYGGNGGN ACNGTNTAYG GNGAYGGNAA  
YYTNGTNTTY

1081 ATHGCNAA YG AYTGGCAYAC NGCNYTNYTN CCNGTNTAYY TNAARGCNTA  
YTAYMGNGAY

20 1141 AAYGGNYTNA TGCARTAYGC NMGNWSNGTN YTNGTNATHC AYAA YATHGC  
NCA YCARGGN

1201 MGNGGNCCNG TNGAYGAYTT YGTNAA YTTY GAYYTNCNG ARCA YTAYAT  
HGAYCA YTTY

1261 AARYTNTAYG AYAA YATHGG NGGNGAYCAY WSNAAYGTNT TYGCNGCNGG  
NYTNAARACN

25 1321 GCNGAYMGNG TNGTNACNGT NWSNAAYGGN TAYATGTGGG ARYTNAARAC  
NWSNGARGGN

1381 GGNTGGGGNY TNCA YGAYAT HATHAAYCAR AAYGAYTGGA ARYTNCARGG  
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30 1441 GGNATHGAYA TGWSNGARTG GAAYCCNGCN GTNGAYGTNC AYYTNCAYWS  
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1501 ACNAAYTAYA CNTTYGARAC NYTNGAYACN GGNAARMGNC ARTGYAARGC  
NGCNYTNCAR

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5 1621 GAYCAYCARA ARGGNGTNGA YATHATHGCN GAYGCNATHC AYTGGATHGC  
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1681 GTNCARYTNG TNATGYTNGG NACNGGNMGN GCNGAYYTNG ARGAYATGYT  
NMGNMGNNTY

10 1741 GARWSNGARC AYWSNGAYAA RGTNMGNMGN TGGGTNGGNT  
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1861 AAYCARYTNT AYGCNATGGC NTAYGGNACN GTNCCNGTNG TNCA YGCNGT  
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15 1921 MGNGAYACNG TNGCNCNTT YGAYCCNTTY AAYGAYACNG GNYTNGGNTG  
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1981 MGNGCNGARG CNAAYMGNAT GATHGAYGCN YTNWSNCAYT  
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20 2041 TAYAARGARW SNTGGMGNGC NTGYMGNGCN MGNGGNATGG  
CNGARGAYYT NWSNTGGGAY

2101 CAYGCNGCNG TNYTNTAYGA RGAYGTNYTN GTNAARGCNA ARTAYCARTG  
GTRRGCAAY

2161 TRRYTNGCNA CNMGNMGNMG NWSNTGYMGN MGNACNTGGA  
CNYTNTTYMG NMGNNTTY

25 2221 WSNYTNCGCNG CNYTNATGMG NGCNWSNCAY YTNMGNMGNM  
CNGAYGGNMGM NMGNNTGGYT

2281 GCNTAYMGNY TNMGNMGNNT NMGNMGNNTN GGNATHHTGGG  
CNGGNACNAT GATGCCNYTN

30 2341 GGNACNGGNM GNGGNGTNGT NTRRTAYGAR ACNGAYGGNG  
AYGGNGAYGA RGCNCAYGGN

2401 ATHTTYCCNY TNATHAAYGG NGARYTNTAY GCNACNYTNA THWSNCCNYT  
NYTNYTINGTN

2461 TTYATHYTNA TGGCNGCN

## CLAIMS

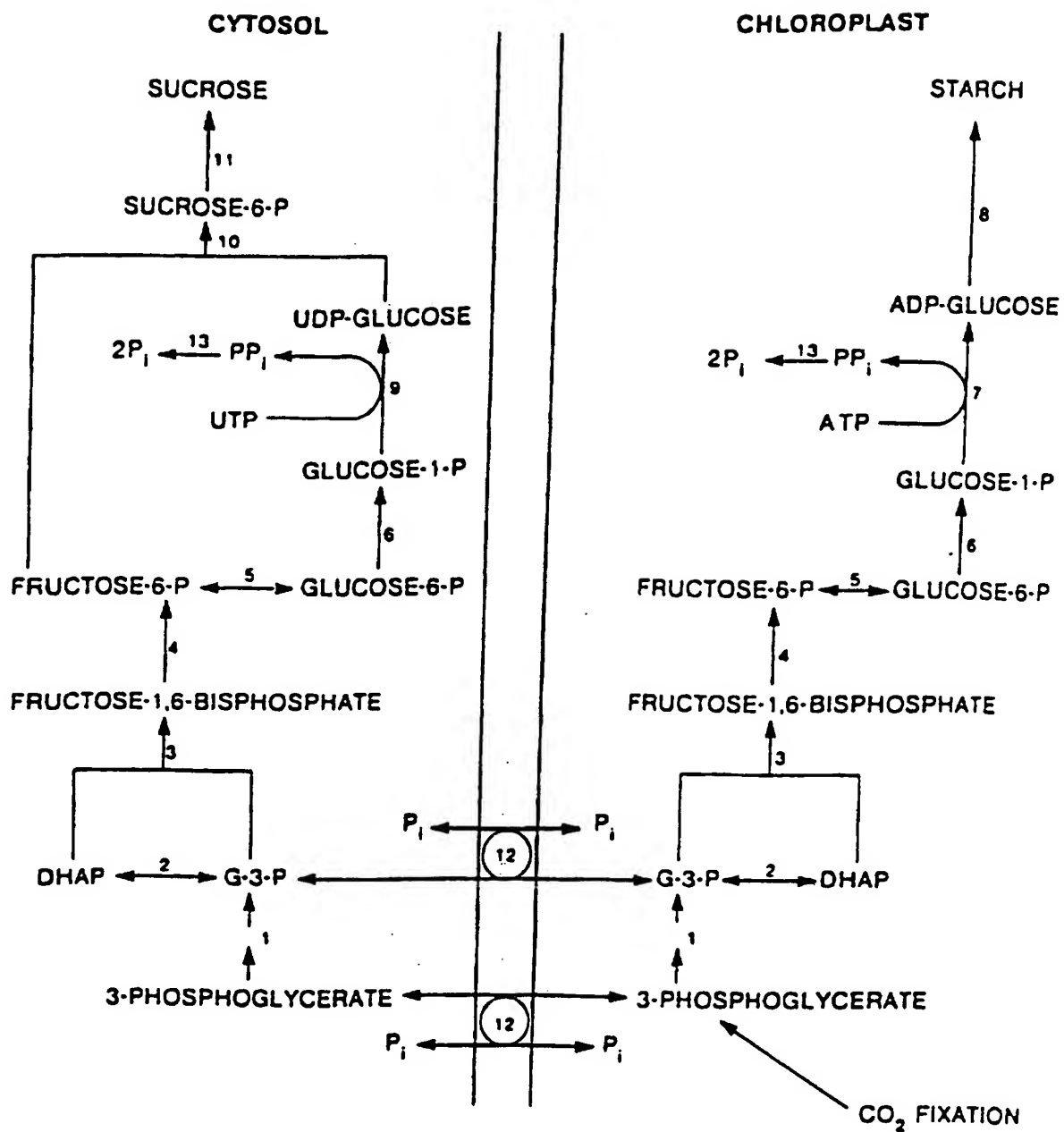
1. A cDNA specifying a soluble starch synthase having the sequences of the inserts in plasmids pSSS6, pSSS10.1 and pSSS6.31 and sequences having sufficient similarity such that when inserted into the genome of an organism which produces starch, the synthesis of starch is altered.  
5
2. The cDNA of the insert of plasmid pSSS6, deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 13th June 1994, under the Accession Number 40651.  
10
3. The cDNA of the insert in plasmid pSSS6.31, deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 22nd August 1994, under the Accession Number NCIMB 40679.  
15
4. The cDNA of the insert in plasmid pSSS10.1, deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 22nd August 1994, under the Accession Number NCIMB 40680.  
20
5. A cDNA, encoding soluble starch synthase which has the sequence SEQ-ID-NO-1, or SEQ-ID-NO-2 or SEQ-ID-NO-3.  
25
6. A transformed plant containing one or more copies of one or more of the said cDNAs claimed in claim 5 in sense or antisense orientation.
7. A method of producing a plant with altered starch synthesising ability comprising stably incorporating into the genome of a recipient plant one or  
30

more than one donor gene specifying soluble starch synthase as claimed in claim 5.

- 5           8.     A method as claimed in claim 7 in which the recipient plant is of the family Gramineae.
9.     A method as claimed in claim 8 in which the recipient plant is of the species *Zea mays*.
- 10       10.    Seeds of a plant as claimed in claim 6.

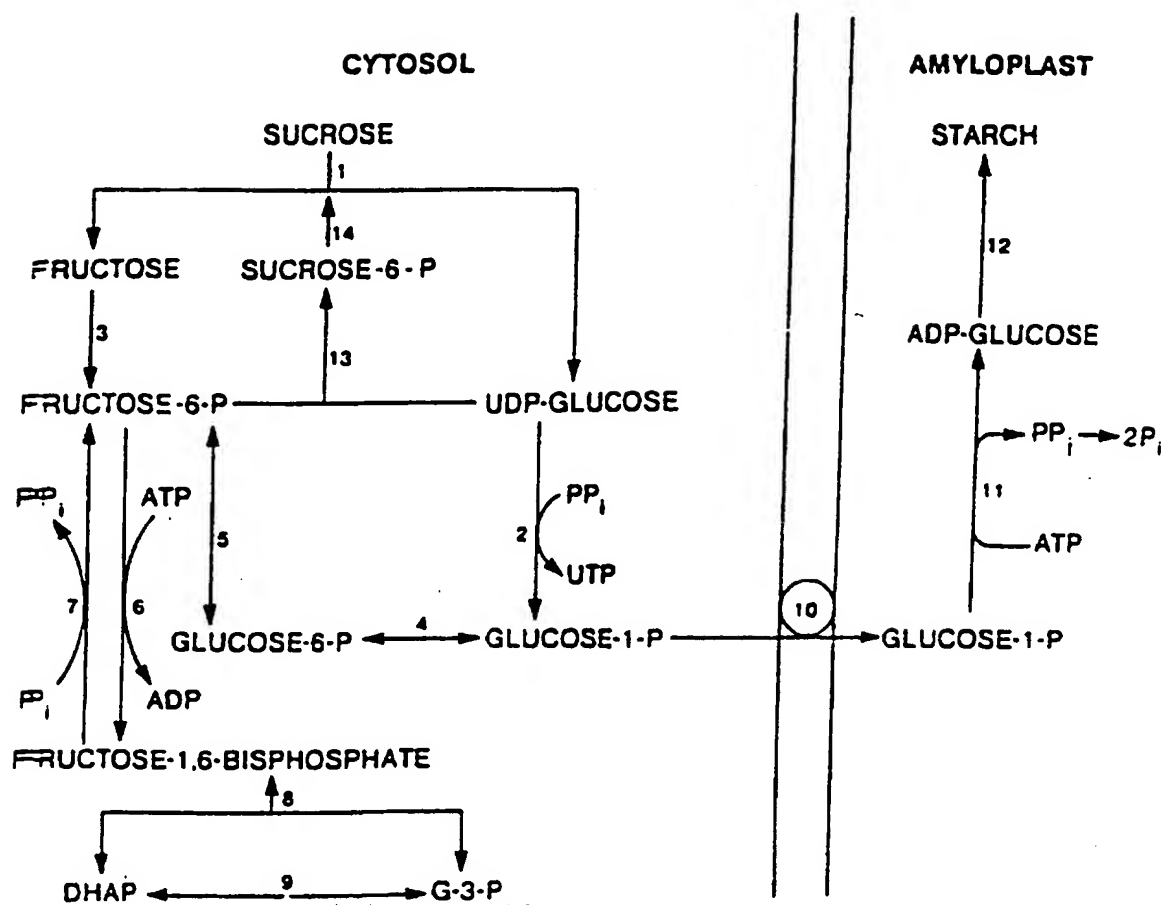
1/2

FIGURE 1



2/2

FIGURE 2





## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>FIVE</u> , line <u>16 - 23 inclusive</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <b>NCIMB</b>	
Address of depositary institution (including postal code and country) <b>23 St Machar Drive, Aberdeen AB1 2RY</b>	
Date of deposit <b>13.06.94</b> <b>22.08.94</b> <b>22.08.94</b>	Accession Number <b>40651</b> <b>40679</b> <b>40680</b>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For receiving Office use only</div> <div style="display: flex; align-items: center; margin-bottom: 10px;"><input type="checkbox"/> This sheet was received with the international application</div> <div style="border-top: 1px solid black; height: 50px; margin-top: 10px;"></div> <div style="border-top: 1px solid black; padding-top: 5px;">Authorized officer</div>	<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For International Bureau use only</div> <div style="display: flex; align-items: center; margin-bottom: 10px;"><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div style="border-top: 1px solid black; height: 50px; margin-top: 10px;"></div> <div style="border-top: 1px solid black; padding-top: 5px;">Authorized officer</div>
--	---

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Seeds,  
Vealotts Hill Research Station,  
Bracknell,  
Berkshire.  
RG12 6EY

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

NAME AND ADDRESS  
OF DEPOSITOR

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  Escherichia coli Sol R containing plasmid pSSS6.31	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40679
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 22 August 1994 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: NCIMB Ltd. 23 St Machar Drive Aberdeen Address:  Date: 23 August 1994	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 23 August 1994

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:

**ACIB Ltd**

Address:

23 St Machar Drive  
Aberdeen Scotland  
UK AB9 1RY

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

  
Date: 23 August 1994

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO  
Zeneca Seeds,  
Jealotts Hill Research  
Station,  
Bracknell, Berkshire. RG12 6EY

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT,  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

NAME AND ADDRESS  
OF DEPOSITOR

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  Escherichia coli Sol R containing plasmid pSSS10.1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCIMB 40680
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by: <div style="margin-top: 10px;"> <input type="checkbox"/> a scientific description                 </div> <div style="margin-top: 10px;"> <input checked="" type="checkbox"/> a proposed taxonomic designation                 </div> <p>(Mark with a cross where applicable)</p>	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on <u>22 August 1994</u> (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on _____ (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: <b>NCIMB Ltd</b> 23 St Machar Drive Aberdeen     Scotland UK     AB9 8QD	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: <u>23 August 1994</u>

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

VIENNA TREATY ON THE INTERNATIONAL  
REGISTRATION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Seeds,  
Jealotts Hill Research Station,  
Bracknell,  
Berkshir .  
RG12 6EY

INTERNATIONAL FORM

VIABILITY STATEMENT  
Issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED

<b>I. DEPOSITOR</b>	<b>II. IDENTIFICATION OF THE MICROORGANISM</b>
Name:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Address: AS ABOVE	NCIMB 40680
	Date of the deposit or of the transfer:
	22 August 1994
<b>III. VIABILITY STATEMENT</b>	
The viability of the microorganism identified under II above was tested on 22 August 1994	
<sup>3</sup> <input checked="" type="checkbox"/> viable	
<sup>3</sup> <input type="checkbox"/> no longer viable	

- <sup>1</sup> Indicate the date of the original deposit or, when a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- <sup>2</sup> In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- <sup>3</sup> Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:

ANDREW SMITH  
DIRECTOR, RESEARCH  
23 St Michael's Lane  
NEWTON

Address:

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Date: 22 August 1994

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Limited  
Zeneca Seeds  
Jealott's Hill Research Station  
Bracknell  
Berkshire  
RG12 6EY

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
Issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
Identified at the bottom of this page

NAME AND ADDRESS  
OF DEPOSITOR

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli Sol R containing plasmid pSSS6	NCIMB 40651
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation	
(Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 13 June 1994 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: 23 St. Andrew's Aberdeen Address: UK	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 14 June 1994

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary  
authority was acquired.

VIENNA TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Limited  
Zeneca Seeds  
Jealott's Hill Research Station  
Bracknell  
Berkshire  
RG12 6EY

INTERNATIONAL FORM

VIABILITY STATEMENT  
Issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
Identified on the following page

NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED

<b>I. DEPOSITOR</b>	<b>II. IDENTIFICATION OF THE MICROORGANISM</b>
Name: As above	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40651
Address:	Date of the deposit or of the transfer:  13 June 1994
<b>III. VIABILITY STATEMENT</b>	
The viability of the microorganism identified under II above was tested on 13 June 1994 <sup>1</sup>	
<sup>2</sup> On that date, the said microorganism was	
<input checked="" type="checkbox"/> <sup>3</sup> viable	
<input type="checkbox"/> <sup>3</sup> no longer viable	

- <sup>1</sup> Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- <sup>2</sup> In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- <sup>3</sup> Mark with a cross the applicable box.



IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:

Address:

23 St Andrew  
Aberdeen

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Date:

*J. MacFarlane*  
14 June 1994

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/GB 96/02990

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/58 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC:

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOL., vol. 108, no. 2 (Suppl.), June 1995, page 50, abstract no. 187, C. HARN ET AL.: "Isolation of a starch synthase cDNA clone from maize W64A" XP000651998 see the abstract. & 1995 annual meeting of the Amer. Soc. of Plant Physiol., Charlotte, N. Carolina, USA, July 29th - August 2nd, 1995.	1-10
X	WO 94 09144 A (ZENECA LTD.) 28 April 1994 see pages 39-41, Example 4 and Claims. --- -/--	1-10



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

17 April 1997

Date of mailing of the international search report

09.05.97

Name and mailing address of the ISA

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Authorized officer

Yeats, S

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 96/02990

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PLANT J., vol. 6, no. 2, 1994, pages 151-159, XP000651922 C. MU ET AL.: "Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm" see the whole document, in particular the summary, Table 1 and the last paragraph of the discussion.</p> <p>---</p>	1-10
X	<p>PLANT PHYSIOL., vol. 103, 1993, pages 565-573, XP000565731 T. BABA ET AL.: "Identification, cDNA cloning, and gene expression of soluble starch synthase in rice (Oryza sativa L.) immature seeds" cited in the application see the abstract and Figure 5.</p> <p>---</p>	1,6,7,10
X	<p>J. BIOL. CHEM., vol. 260, 1985, pages 16451-16459, XP000652758 M.D. MARKS ET AL.: "Nucleotide sequence analysis of zein mRNAs from maize endosperm" see abstract and Figure 4, clone cZ19C1.</p> <p>-----</p>	1
A	<p>EP 0 521 621 A (ADVANCED TECHNOLOGIES (CAMBRIDGE) LTD.) 7 January 1993 see the claims.</p> <p>---</p>	1,6,7
P,X	<p>WO 96 15248 A (INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH) 23 May 1996 see Examples 1-12 and Claims.</p> <p>-----</p>	1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB 96/02990

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9409144 A	28-04-94	AU 2696492 A EP 0664835 A	09-05-94 02-08-95
EP 521621 A	07-01-93	CA 2071010 A US 5365016 A	13-12-92 15-11-94
WO 9615248 A	23-05-96	DE 4441408 A AU 3927995 A	15-05-96 06-06-96